Human Thrombin/Antithrombin Complex Antigen ELISA Kit

Catalog No. CKH016A **Size:** 1 x 96 tests
CKH016B **Size:** 5 x 96 tests

Intended Use:

This human Thrombin/Antithrombin Complex assay is intended for the quantitative determination of the covalent complex of thrombin and its inhibitor antithrombin III in human plasma and serum.

Background:

Antithrombin III is a glycosylated plasma serine protease inhibitor that forms a stoichiometric complex with coagulation cascade enzymes. Thrombin is a two chain vitamin K-dependent glycosylated serine protease that is activated from prothrombin in the coagulation cascade. Antithrombin III inhibits thrombin with heparin enhanced kinetics and forms a 1:1 covalent complex.

Assay Principle:

Human TAT complex in samples will bind to the antihuman thrombin capture antibody coated on the microtiter plate. After appropriate washing steps, monoclonal anti-human antithrombin primary antibody binds to TAT complex captured on the plate. Excess antibody is washed away and bound monoclonal antibody is then reacted with the secondary antibody conjugated to horseradish peroxidase. TMB substrate isused for color development at 450nm. A standard calibration curve is prepared along with the samples to be measured using dilutions of TAT complex. Color development is proportional to the concentration of TAT complex in the samples. Free thrombin, prothrombin and antithrombin III will not be detected by this assay.

Reagents Provided:

Item A: 96-well antibody coated microtiter strip plate
8 x 12 removable well strips containing Anti-Human Thrombin capture antibody, blocked, and dried on the surface.

Item B: 10X Wash Buffer
1 bottle of 50 ml; bring to 1X using DI water

Item C: Human Thrombin/Antithrombin Complex standard
1 vial of lyophilized standard

Item D: Anti-Human Antithrombin III primary antibody
1 vial of lyophilized polyclonal antibody

Item E: Anti-mouse horseradish peroxidase secondary antibody
1 vial of concentrated HRP labeled antibody

Item F: TMB substrate solution
1 bottle of 10 ml solution

Storage and Stability:

All kit components must be stored at 4°C. Store unopened plate and any unused microtiter strips in the pouch with desiccant. Reconstituted standard and primary antibody may be stored at -80°C for later use. **DO NOT** freeze/thaw the standard and primary antibody more than once. All other unused kit components must be stored at 4°C. Kit should be used no later than the expiration date.

Reagents and Equipment Required:

- Microtiter plate shaker capable of 300 rpm uniform horizontally circular movement
- Manifold dispenser/aspirator or automated microplate washer
- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes and Pipette tips
- Deionized or distilled water
- Polypropylene tubes for dilution of standard
- Paper towels or laboratory wipes
- 1 N H_2SO_4 or 1 N HCl
- Bovine Serum Albumin Fraction V (BSA)
- Tris(hydroxymethyl)aminomethane (Tris)
- Sodium Chloride (NaCl)

Warnings:

Avoid skin and eye contact when using TMB substrate solution, because it may be irritating to eyes, skin, and respiratory system. Wear safety goggles and gloves.

Precautions:

- Do not mix any reagents or components of this kit with any reagents or components of any other kit. This kit is designed to work properly as provided.
- Always pour peroxidase substrate out of the bottle into a clean test tube.
- Do not pipette out of the bottle as contamination could result.
- Keep plate covered except when adding reagents, washing, or reading.
- Do not pipette reagents by mouth and avoid contact of reagents and specimens with skin.
- Do not smoke, drink, or eat in areas where specimens or reagents are being handled.

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Preparation of Reagents:

TBS buffer: 0.1 M Tris, 0.15 M NaCl, pH 7.4

Blocking buffer (BB): 3% BSA (w/v) in TBS

1x Wash buffer: Dilute 50 ml of the 10x Wash Buffer Concentrate with 450 ml of deionized water..

Sample Collection:

Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 mins at 1,000 x g within 30 minutes of collection. Assay immediately or aliquot and store at $\leq$ -20°C. Avoid repeated freeze-thaw cycles.

Assay Procedure:

Perform assay at room temperature.

Vigorously shake plate (300 rpm) at each step of the assay.

Preparation of Standard:
Reconstitute standard by adding 1 ml of Blocking Buffer directly to the vial and agitate gently to completely dissolve contents. This will result in a 1000 ng/ml standard solution.

Dilution Table for preparation of Human TAT Complex Standard

TAT Complex Concentration (ng/ml)	Dilutions
1000	From Standard Vial
500	500 µl (BB) + 500 µl (1000 ng/ml)
250	500 µl (BB) + 500 µl (500 ng/ml)
100	600 µl (BB) + 400 µl (250 ng/ml)
50	500 µl (BB) + 500 µl (100 ng/ml)
25	500 µl (BB) + 500 µl (50 ng/ml)
10	600 µl (BB) + 400 µl (25 ng/ml)
5	500 µl (BB) + 500 µl (10 ng/ml)
2	600 µl (BB) + 400 µl (5 ng/ml)
1	500 µl (BB) + 500 µl (2 ng/ml)
0	500 µl (BB) Zero point to determine background.

Important Note: Dilutions for the standard curve and zero standard must be made and applied to the plate immediately.

Standard and Unknown Addition:
Remove microtiter plate from bag and add 100 µl TAT Complex Standards (in duplicate) and unknowns to wells. Carefully record position of standards and unknowns. Shake plate at 300 rpm for 30 minutes. Wash wells three times with 300 µl wash buffer. Remove excess wash by gently tapping plate on paper towel or Kimwipe.

NOTE: The assay measures Total Human TAT Complex in the 1-1000 ng/ml range. Samples giving human TAT complex levels above 1000 ng/ml should be diluted in blocking buffer before use. Normal human plasma samples should be applied directly to the plate without dilution. A 1:500-1:1000 dilution for normal human serum is suggested for best results.

Primary Antibody Addition:
Add 10 ml of blocking buffer directly to the primary antibody vial and agitate gently to completely dissolve contents. Add 100 µl to all wells. Shake plate at 300 rpm for 30 minutes. Wash wells three times with 300 µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

Secondary Antibody Addition:
Briefly centrifuge vial before opening. Dilute 2 µl of conjugated secondary antibody in 10 ml of blocking buffer and add 100 µl to all wells. Shake plate at 300 rpm for 30 minutes. Wash wells three times with 300 µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

Substrate Incubation:
Add 100 µl TMB substrate to all wells and shake plate for 2-10 minutes. Substrate will change from colorless to different strengths of blue. Quench reaction by adding 50 µl of 1N H_2SO_4 or HCl stop solution to all wells when samples are visually in the same range as the standards. Add stop solution to wells in the same order as substrate upon which color will change from blue to yellow. Mix thoroughly by gently shaking the plate and read final absorbance values at 450nm. For best results read plate immediately.

Measurement:
Set the absorbance at 450 nm in a microtiter plate spectrophotometer. Measure the absorbance in all wells at 450 nm. Subtract zero point from all standards and unknowns to determine corrected absorbance (A_{450}).

Assay Calibration:
Plot A_{450} against the amount of TAT Complex in the standards. Fit a straight line through the linear points of the standard curve using a linear fit procedure if unknowns appear on the linear portion of the standard curve. Alternatively, create a standard curve by analyzing the data using a software program capable of generating a four parameter logistic (4PL) curve fit. The amount of human TAT Complex in the unknowns can be determined from this curve. If the samples have been diluted, the calculated concentration must be multiplied by the dilution factor.

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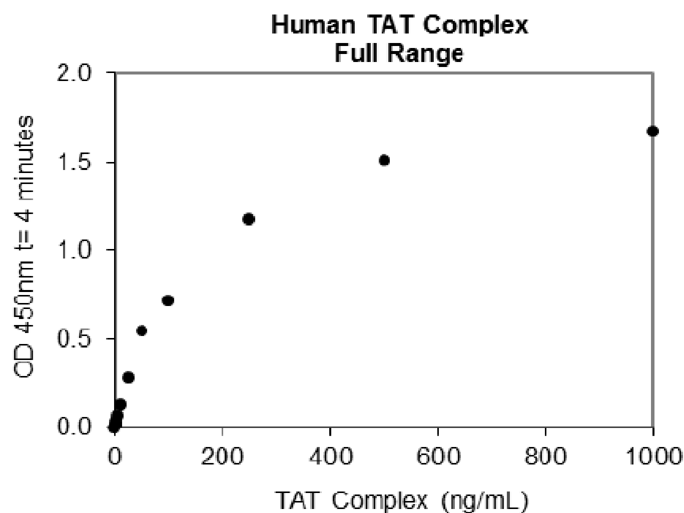
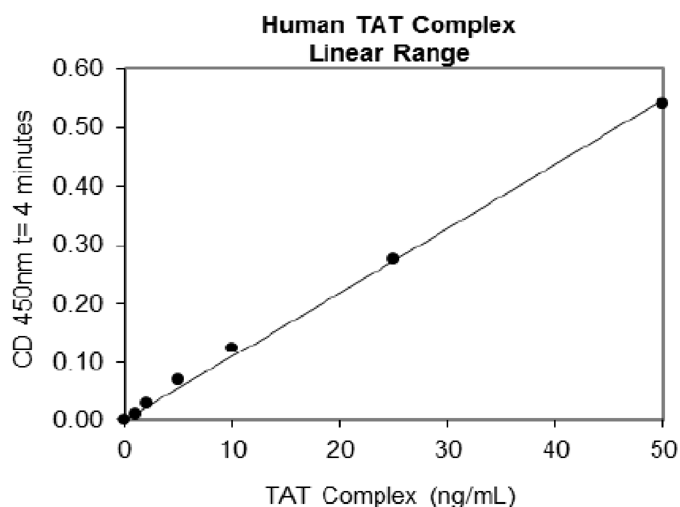
Typical Standard Curve
(EXAMPLE ONLY, DO NOT USE)





Expected Values:
Concentration of TAT complex in normal human plasma has been found to be 2.1 ng/ml and was increased in deep vein thrombosis (9.4 ng/ml) and disseminated intravascular coagulation (15.8 ng/ml).

Sensitivity:
The minimum detectable dose (MDD) was determined by adding two standard deviations to the mean optical density value of 20 zero standard replicates (range OD450: 0.099-0.117) and calculating the corresponding concentration. The MDD was 0.718 ng/ml.

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Specificity:
This assay recognizes natural human TAT complex. Natural human thrombin, prothrombin, and antithrombin III were assayed for cross-reactivity. No significant cross-reactivity was observed.

Sample Values:
Samples were evaluated for the presence of the antigen at varying dilutions.

Sample Type	Dilution	Mean (µg/mL)
Citrate Plasma	Undiluted	0.01
Serum	1:320	52
	1:640	53

Example of ELISA Plate Layout
96 Well Plate: 22 Standard wells, 74 Sample wells

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	25 ng/ml	50 ng/ml	100 ng/ml	250 ng/ml	500 ng/ml	1000 ng/ml	
B	0	1 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	25 ng/ml	50 ng/ml	100 ng/ml	250 ng/ml	500 ng/ml	1000 ng/ml	
C												
D												
E												
F												
G												
H												

NOT FOR HUMAN USE. FOR RESEARCH ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE.

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